A HISTOCHEMICAL STUDY OF DIGESTION AND DIGESTIVE ENZYMES IN THE RHYNCHOCOELAN LINEUS RUBER (O. F. MÜLLER)

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It has been shown in a previous account (Jennings, 1960) that digestion in the rhynchocoelan *Lineus ruber* is the result of both extracellular and intracellular processes. The food, which consists of animals such as small annelids and crustaceans captured by means of the eversible proboscis, is swallowed whole and, after being killed by acid secretions poured on to it during its passage through the foregut, is broken down into a semi-fluid mass within minutes of arrival in the intestine. The enzyme bringing about this initial, extracellular, breakdown is produced by gland cells scattered throughout the intestinal gastrodermis and operates at a pH of 5.0–5.5. The fragmenting food is phagocytosed by other gastrodermal cells and digestion completed intracellularly.

In the present work the course of digestion has been examined in greater detail and an attempt made to identify some of the enzymes concerned in both the breakdown of the food and the general metabolic activity of the gastrodermis.

Materials and Methods

Individual *Lineus ruber* were isolated and starved for seven days to clear the gut of all traces of previous meals. Individual isolation was necessary since after five or six days without food cannibalism often occurs.

To study the course of digestion, and to locate and identify the enzymes concerned, starved *Lineus* were fed upon inert test foods such as clotted frog blood, either alone or mixed with cooked beef fat or starch paste. These foods were used in preference to the natural living food to eliminate any possibility of enzymes contained in the latter being mistaken for those produced by the *Lineus* gut.

Series of *Lineus* were killed for examination after seven days' starvation and at progressive intervals up to 48 hours after an observed meal on one or other of the test foods. In earlier experiments the *Lineus* were killed by freezing in isopentane cooled by liquid nitrogen to $-160°$ C. and subsequently dehydrated for 48 hours at $-40°$ C. under a vacuum of 10^{-3} mm. mercury. Such specimens were embedded in paraffin wax (melting point 42° C.), sectioned at 8 μ and examined by the histochemical techniques listed below. During the course of the work, however, it was found that specimens fixed for 12 hours at 4° C. in 10% formalin in sea water, buffered to pH 7.0, and then rapidly dehydrated in absolute acetone at the same temperature, cleared in xylol at room temperature and embedded in 42° C. wax showed no significant decrease in enzyme activity when compared with the freeze dried specimens. The duration of dehydration, clearing and

embedding was kept to the absolute minimum consistent with the size of the specimen. This method of preparing sections for histochemical examination was adopted for the bulk of the work as it enabled many more specimens to be dealt with in a given time and was far less troublesome in operation.

The sections were mounted with albumen and after drying at 20° C. for six hours were dewaxed in xylol and passed through two changes of absolute acetone before being transferred directly into the various reagents for visualising enzyme activity.

Carbonic anhydrase activity, known to be associated with production of hydrochloric acid in the mammal stomach, was visualised by the cobalt sulphate-bicarbonate method given by Hausler (1958). Sections were incubated for three hours at 20° C. and it was found that for optimum results the layer of substrate solution upon the sections must not exceed 1 mm. in depth. The medium was buffered to pH 8.0 and control sections incubated in the presence of 4×10^{-3} *M* Diamox sodium (2 acetylamino-1,3,4-thiadiazole-5-sulfonamide sodium), a specific inhibitor for carbonic anhydrase. As a further control, and to check the reliability of the method, sections of mouse stomach were similarly treated.

Proteolytic enzymes were investigated by the methods of Hess and Pearse (1958) for cathepsin C type enzymes and Burstone and Folk (1956) for leucine aminopeptidase. To detect cathepsin C type activity, sections were transferred from acetone into a 10^{-5} *M* solution of E-600 (diethyl-p-nitrophenyl phosphate) to inactivate esterases whose presence would otherwise give false positive reactions. The sections were then incubated for three to four hours at 20° C. in a standard indoxyl acetate medium buffered at pH 5.0. Control sections were immersed for one hour, before incubation, in 1×10^{-3} *M* cysteine solution which activates cathepsin C so that sections treated in this way show a more intense enzymatic action than do non-treated ones. Further controls were performed by immersing sections in 1×10^{-3} *M* lead nitrate solution for one hour, or in water at 90° C. for three minutes, before incubation. For leucine aminopeptidase activity sections were incubated for six hours at 20° C. in a medium containing L-leucyl-β-naphthylamide as substrate and Garnet G.B.C. as a simultaneous coupler. The medium was buffered to pH 7.2 and heat-inactivated sections used as controls.

Lipolytic activity was investigated by the method of Gomori (1952) using Tween 80 as the substrate in a medium buffered to pH 7.2. Sections of *Lineus* fed on blood mixed with beef fat were incubated for twelve hours at 20° C. and again heat-inactivated sections were used as controls.

Attempts were made to detect carbohydrase activity by using the ferric-8-hydroxyquinoline method for β-glucuronidase as modified by Billett and McGee-Russell (1955). This method failed to give satisfactory results and observations on carbohydrate digestion were limited to tracing the fate of starch meals by the Lugol's iodine technique.

Alkaline phosphatase activity was demonstrated by the calcium phosphate method (Gomori, 1952). Sections were incubated for two hours at 20° C. in a medium containing sodium β-glycerophosphate buffered to pH 8.0 and control sections inactivated by heat prior to incubation. Sections were also examined for acid phosphatase activity (Gomori, 1952), again using sodium β-glycerophosphate as substrate.

Observations

Histology of the gut

The histological structure of the gut in *Lineus* has been described in detail elsewhere (Jennings, 1960). Briefly, the gut consists of three regions, the mouth and buccal cavity, the foregut, and the intestine. It is ciliated throughout its length and lacks both multicellular glands and musculature. The buccal cavity is lined by ciliated cuboidal cells backed by masses of acidophil and basophil gland cells, the majority of which are Alcian blue- and periodic acid-Schiff-positive and produce mucus to facilitate ingestion. The foregut is lined by similar ciliated cells, interspersed with acidophil gland cells, lying upon tissue with indistinct cell walls containing numerous acidophil and basophil glands, many of which are mucus-producing. The intestine forms the major portion of the gut and bears paired serially repeated lateral pouches throughout its length. The intestinal wall, or gastrodermis, is made up of two types of cells which stand in a single layer upon a thin basement membrane. The larger and more numerous cells are columnar and the cytoplasm usually contains phagocytosed food particles in various stages of digestion. The second type of cell is glandular and contains up to 30 acidophil proteinaceous spheres which are discharged into the gut lumen when food enters.

Enzymes produced in the gut

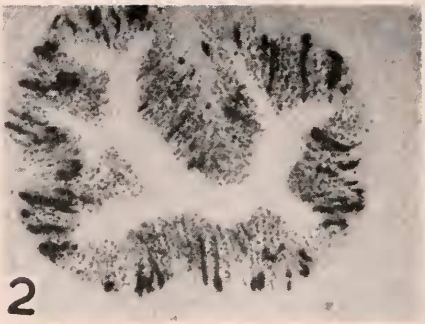
Carbonic anhydrase

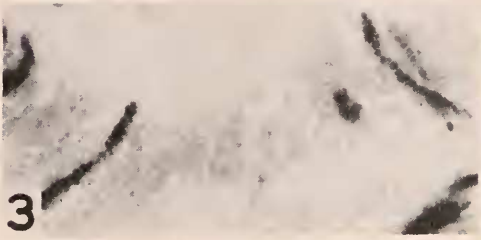
Carbonic anhydrase activity was found in some 10–15% of the acidophil gland cells of the buccal cavity and foregut (Fig. 1). In the latter, gland cells in both the lining epithelium and the backing syncytial tissue gave positive reactions. The number of reactive cells, and the degree of activity, could not be related to the time of feeding and it would appear that the enzyme is always present in an active condition. Control sections incubated in the presence of the specific inhibitor, Diamox sodium, gave no reaction. Sections of mouse stomach incubated at 37° C. showed intense activity in the acidophil oxyntic cells of the fundic glands, which are known to be the source of the gastric hydrochloric acid. This activity, as in *Lineus*, was inhibited by Diamox sodium.

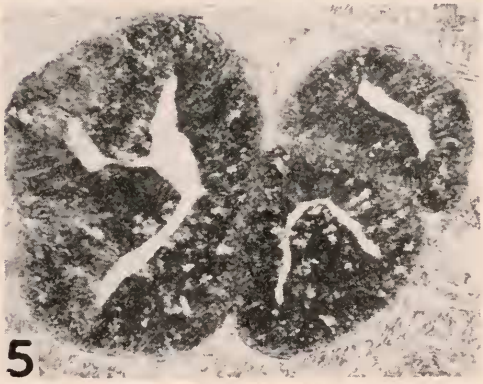
Cathepsin C

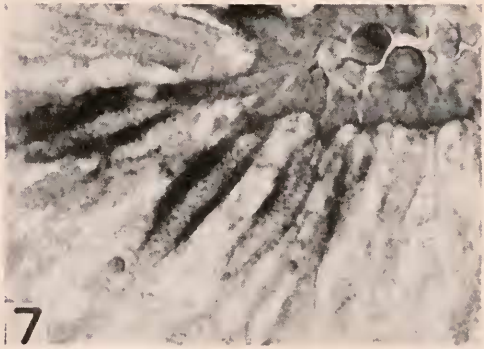
During starvation the gland cells of the gastrodermis give an intense positive reaction to the Hess and Pearse method for cathepsin C (Figs. 2 and 3). The individual spheres within the gland cells stained so intensely that often other details of the cell, such as the nucleus, were obscured. Sections immersed in 10^{-3} *M* cysteine solution, a known activator of cathepsin C type enzymes, before incubation, reached the maximum density of staining in approximately half the incubation time needed by non-activated sections. Sections inactivated by heat or by immersion in 10^{-3} *M* lead nitrate solution gave no reaction whatsoever.

Sections prepared within 30 minutes of feeding showed that the majority of the gland cells had discharged their spheres and such cells failed to give any reaction. This condition (Fig. 4) persisted for two to three hours and then the









FIGS. 1–7.

gland cells gradually filled up again with active spheres so that about six hours after feeding they were back in their normal condition. After a meal of frog blood haemolysis occurred as the food entered the intestine, and within 30 minutes the digesting mass in the lumen gave a fairly strong reaction for cathepsin C. About this time the columnar cells of the gastrodermis commenced phagocytosis of the food and the phagocytosed material continued to give a cathepsin reaction within the cells (Fig. 4). This, however, decreased rapidly with time and it was clear that the reaction was the result of enzyme being phagocytosed with the food and remaining active for a short time within the cells. There was no evidence of intracellular production of cathepsin C. One and a half hours after feeding, the gastrodermis was loaded with phagocytosed material and the apparently intracellular cathepsin activity had disappeared.

The optimum pH for visualisation of the enzyme was 5.0 and this agrees with previous *in vivo* observations using indicator-stained food, which showed that the early stages of digestion went on at pH 5.0–5.5 (Jennings, 1960).

Leucine aminopeptidase

Sections of starved *Lineus*, and of specimens fixed within one and a half hours of feeding, gave no reaction for leucine aminopeptidase. About two hours after feeding, however, phagocytosed material started to show a faint positive reaction. The intensity of the reaction increased rapidly with time, and six hours after feeding all the material in the columnar cells of the gastrodermis gave an extremely strong and vivid reaction (Fig. 5). Heat-inactivated control sections showed no such activity. At no time was any leucine aminopeptidase activity found in either

FIGURE 1. Longitudinal section of the foregut in *Lineus*, showing carbonic anhydrase activity in the gland cells of the ciliated epithelium. Hausler cobalt sulphate-bicarbonate method. Scale: 1 cm. = 50 μ.

FIGURE 2. Transverse section of an intestinal pouch in a starved *Lineus*, showing the positive cathepsin C type reaction given by the gland cells of the gastrodermis. The gland cells are seen as dark streaks. Hess and Pearse E600-indoxyl acetate method. Scale: 1 cm. = 50 μ.

FIGURE 3. Transverse section of a portion of the gastrodermis of a starved *Lineus*, showing two gland cells packed with enzymatic spheres giving an intense cathepsin C type reaction. Hess and Pearse method. Scale: 1 cm. = 25 μ.

FIGURE 4. Longitudinal section of the gastrodermis in *Lineus* 30 minutes after feeding. Hess and Pearse method. The gland cells have discharged their spheres and are no longer apparent by this technique. The columnar cells have commenced phagocytosis, and the newly engulfed food, seen in the distal regions of the cells, shows cathepsin C type activity retained from the initial extracellular phase of digestion. Scale: 1 cm. = 50 μ.

FIGURE 5. Transverse section of two intestinal pouches in *Lineus* six hours after feeding. The gastrodermis is swollen and loaded with food vacuoles all exhibiting intense leucine aminopeptidase activity. Burstone and Folk L-leucyl-β-naphthylamide G.B.C. method. Scale: 1 cm. = 50 μ.

FIGURE 6. Transverse section of an intestinal pouch in *Lineus* four hours after a meal of frog blood and beef fat. The gastrodermis is loaded with food vacuoles, many of which show lipolytic activity (seen as black spheres). Gomori Tween 80-lead sulphide method. Scale: 1 cm. = 50 μ.

FIGURE 7. Transverse section of a portion of the *Lineus* gastrodermis within 5 minutes of feeding, showing intense alkaline phosphatase activity around the gland cells. The gut lumen (top right) contains haemolysing frog erythrocytes. Gomori calcium phosphate method. Scale: 1 cm. = 25 μ.

the gland cells of the gastrodermis or the gut lumen, and this enzyme would appear to be entirely intracellular and to be concerned only in the later stages of digestion. The optimum pH for the visualisation was 7.2 and this difference in pH optima between the lumen-acting cathepsin C and the intracellular aminopeptidase is probably the reason why aminopeptidase activity does not commence immediately phagocytosed material enters the cells. It has been seen that newly phagocytosed food continues to show cathepsin C activity for over one hour after feeding and so is presumably still at, or near, the acidic pH value necessary for this. The decrease of cathepsin C activity and its gradual replacement by aminopeptidase will be accompanied by an increase in pH value up to the optimum 7.2 and this apparently takes a little time to achieve.

Leucine aminopeptidase activity continues whilst food remains in the gastrodermis and finally disappears 9 to 12 hours after feeding, depending upon the amount of food taken. It would appear, therefore, that this enzyme is normally present in an inactive form, unlike the cathepsin C of the gland cells, and only becomes active (*i.e.*, as shown by the present method) when it is secreted from the cytoplasm into a food vacuole.

Lipase

Small amounts of lipolytic activity were found in the gastrodermis and parenchyma during all stages of starvation. These were probably the result of the starved *Lineus* utilising its fat reserves which are laid down in these sites (Jennings, 1960).

No significant increase in activity occurred until two to three hours after a meal of blood and beef fat. About this time a few food vacuoles resulting from phagocytosis of the meal showed a positive reaction. The number of such vacuoles then increased rapidly and within a further hour they could be found throughout the gastrodermis (Fig. 6). As intracellular digestion progressed the number of reactive vacuoles diminished but some could be found up to 48 hours after feeding. This was a consequence, no doubt, of the unusually large proportion of fat in the meal.

The optimum pH for the reaction was 7.2–7.4 and again, as in the case of aminopeptidase, this is probably the reason for the time lag between the onset of phagocytosis and the appearance of lipolytic activity.

Control sections inactivated by heat showed no lipolysis.

Carbohydrase activity

Attempts to localise β-glucuronidase activity by the Billett and McGee-Russell method during starvation and after meals of blood and starch paste were unsuccessful. The end product of the histochemical reaction, ferric-8-hydroxyquinoline, was precipitated indiscriminately over the entire sections, both experimental and control. Sections stained with Lugol's iodine, however, showed progressive digestion and disappearance of phagocytosed starch parallel in time with the aminopeptidase and lipase activity, and it was concluded that the unknown carbohydrases act at a similar slightly alkaline pH. There was no extracellular carbohydrate digestion.

Alkaline phosphatase

The gland cells of the gastrodermis normally show no alkaline phosphatase activity but immediately after the *Lineus* has fed, when they are discharging their enzymatic spheres into the gut lumen, intense alkaline phosphatase activity appears around the cell walls (Fig. 7). This localised activity disappears during reconstitution of the gland cells and so appears to be associated with their secretory rather than recovery phase.

The columnar cells of the gastrodermis show at all times intense alkaline phosphatase activity along their free ciliated borders (Fig. 8). The zone of activity varies in depth from a narrow band 3–5 μ deep immediately below the cilia, to a broad belt which may extend down into the cells for as much as one-third of

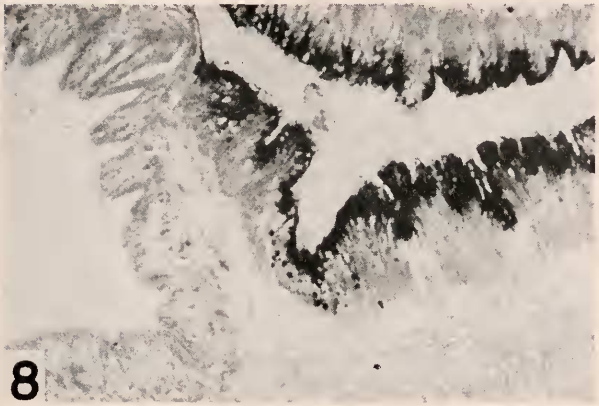


FIGURE 8. Longitudinal section of a portion of the foregut (left) and intestine (right) of a starved *Lineus*. Gomori method for alkaline phosphatase. The cells of the foregut show no activity, in marked contrast to those of the intestinal gastrodermis which show intense activity distally. Scale: 1 cm = 50 μ.

FIGURE 9. Transverse section of an intestinal pouch of *Lineus* four hours after feeding. All the food vacuoles and some of the cytoplasm surrounding them show intense alkaline phosphatase activity. Gomori method. Scale: 1 cm. = 50 μ.

their depth. This activity is obviously concerned with some aspect of the digestive function of the gastrodermal cells, for it is completely absent from the cells of the foregut wall (Fig. 8).

The distal band of alkaline phosphatase activity persists during extracellular digestion but as phagocytosis and intracellular digestion advance, it spreads downwards until the entire cytoplasm of the columnar cells gives a positive reaction. The activity is more concentrated around and within the food vacuoles (Fig. 9) and reaches its peak at about the same time as aminopeptidase and lipase activity. Thus it would appear to be connected with the secretion of these enzymes and the absorption of the products of intracellular digestion. This condition persists until digestion is completed and then the activity decreases in amount until only the normal distal zone remains.

Acid phosphatase

No trace of acid phosphatase activity was found in any region of the gut

Discussion

Whilst it is clear that many more enzymes than the ones located in this work will be concerned in digestion in *Lineus*, sufficient information has been obtained for the sequence of digestive processes and the part played by the different types of enzymes to be understood.

The presence of carbonic anhydrase in acidophil gland cells of the buccal cavity and foregut would suggest that these cells are the source of the acid secretions poured on to the food during ingestion to assist in killing it and to provide a medium of suitably low pH value for the initial stages of digestion, taking into account the known association of this enzyme with hydrochloric acid secretion in the oxyntic cells of the mammal. The inhibition of the enzyme by the specific inhibitor for carbonic anhydrase and the similar results obtained with mouse oxyntic cells leave little doubt as to its identity. It was suggested previously that certain basophils in the foregut wall produced the acid secretions (Jennings, 1960) but since these fail to show carbonic anhydrase activity it may be that they have some other, unknown, function.

The early, extracellular, stages of digestion are effected by the proteolytic enzyme discharged from the gland cells of the gastrodermis. This has been identified as cathepsin C, or, at least, a cathepsin C *type* enzyme, and the identification confirmed by use of specific activators and inhibitors. Cathepsin C is an endopeptidase and attacks inner portions of protein chains to break down the molecule into simpler polypeptides and peptides. Thus its function in *Lineus* is to initiate proteolysis and break down the food to a condition suitable for entry into the gut cells where digestion is completed. In this respect the enzyme has an adaptive significance comparable to that of the elaboration of the feeding mechanism in the triclad Turbellaria, where purely mechanical means are used to make the food available for phagocytosis, and extracellular digestion does not occur (Jennings, 1957). In *Lineus*, and presumably most rhynchocoelans, a simpler type of feeding mechanism means that the food is swallowed whole and consequently there must be some other provision for its breakdown before intracellular digestion can begin.

The intracellular proteolysis appears to be effected by aminopeptidases, of which one example, leucine aminopeptidase, has been identified. These enzymes are exopeptidases and remove terminal amino acids from polypeptides resulting from endopeptidase activity and so complete digestion of the protein content of the food. They function at a slightly alkaline pH, in contrast to the extracellularly-acting endopeptidase, cathepsin C, which requires a fairly strongly acidic medium. Thus proteolysis in *Lineus* resembles that in most other animals in that it occurs in two distinct phases, the first acidic, the second alkaline.

The extracellular and intracellular proteolysis makes the fat and carbohydrate content of the food available for digestion by breaking down tissue and cell membranes, and the digestion of these food elements is entirely intracellular. Lipolytic activity appears at about the same time as aminopeptidase and the enzyme responsible operates at a similarly slightly alkaline pH. It is probable that the enzyme visualized here is the "true lipase" of Gomori (1952), homologous with mammalian pancreatic lipase, since an unsaturated substrate (Tween 80) was used and this, according to Gomori, is attacked only by pancreatic type lipase and not by other esterases.

It was not possible to identify any carbohydrases but the intracellular digestion of starch showed that these are present and working at a pH similar to that needed for aminopeptidase and lipase activity. Rosenbaum and Rolon (1960) located β-glucuronidase activity in the phagocytic gut cells of the not too distantly related triclad flatworm and it may be that this enzyme is also present in *Lineus* but failed to survive fixation.

The intense alkaline phosphatase activity observed in the distal region of the columnar gastrodermal cells appears to be related to the part played by the cell wall and its cilia in the phagocytic uptake of food. The cilia coalesce into pseudopodia-like processes which engulf the fragmenting food (Jennings, 1960) and the alkaline phosphatase no doubt plays some part in this modification and later recovery of the cilia. This interpretation is supported by the absence of phosphatase activity from the foregut cells, which are not concerned in uptake of food and whose cilia show no such modification. The alkaline phosphatase activity developing deeper within the gastrodermal cells during intracellular digestion would seem to be related to secretion from the cytoplasm into the food vacuoles of aminopeptidases, lipase and carbohydrases since these appear simultaneously with the phosphatase.

Summary

1. Digestion in the rhynchocoelan *Lineus ruber* is both extracellular and intracellular. The extracellular phase is entirely proteolytic and is brought about by an endopeptidase acting in an acid medium. The semi-digested food is then phagocytosed and digestion is completed in the second, intracellular phase by exopeptidases, lipases and presumed carbohydrases, all operating in an alkaline medium.

2. The following enzymes have been located and identified by histochemical methods: carbonic anhydrase, a cathepsin C type protease (endopeptidase), leucine aminopeptidase (exopeptidase), lipase and alkaline phosphatase.

3. Carbonic anhydrase occurs in acidophil gland cells in the buccal cavity and foregut. It is believed to be associated with production of acid used to kill the food and provide a suitable medium for the extracellular phase of digestion.

4. The cathepsin C type protease is produced by gland cells in the gastrodermis and is discharged into the gut lumen to bring about the initial extracellular proteolysis.

5. Leucine aminopeptidase is produced within the phagocytic cells of the gastrodermis when food vacuoles are present and is concerned in completion of protein digestion.

6. Lipase, identified as "true lipase" homologous with mammalian pancreatic lipase, is formed within the phagocytic cells at the same time as leucine aminopeptidase and attacks the fat content of the food.

7. Alkaline phosphatase is present in an active form in the gastrodermis and appears to be concerned with phagocytosis. The amount present increases when intracellular digestion occurs and this increase is apparently associated with secretion of aminopeptidase and lipase into the food vacuoles.

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